Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol

Supplemental Assay Method for Conducting the Hemagglutination Inhibition Assay for Equine Influenza Antibody

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Supplemental Assay Method for Conducting the Hemagglutination Inhibition Assay for Equine Influenza Antibody

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Supplemental Assay Method for Conducting the Hemagglutination Inhibition Assay for Equine Influenza Antibody

1. Introduction

1.1 Background

This Supplemental Assay Method (SAM) describes an *in vitro* assay method for determining the hemagglutination inhibition (HI) antibody titer of sera from guinea pigs vaccinated with type A equine influenza viruses as part of a potency test for veterinary vaccines.

Note: For this SAM, the dilution terminology of 1:10, 1:20, etc., specifies 1 part plus 9 parts (liquid), 1 part plus 19 parts, etc.

1.2 Keywords

Hemagglutination inhibition, HI, potency test, equine influenza, serology, *in vitro*

2. Materials

2.1 Equipment/instrumentation

2.1.1 Micropipettors: 200 µl and 1000 µl single channel,[1] 5-50 µl x 12 channel,[2] and tips[3]

2.1.2 Centrifuge[4] and rotor[5]

2.1.3 Vortex mixer[6]

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[1] Pipetman®, Rainin Instrument Co., Mack Rd., Box 4026, Woburn, MA 01888 or equivalent
[2] Finnpipettes®, Cat. No. NX204662D, A. Daigger Company, Inc., 199 Carpenter Ave., Wheeling, IL 60090 or equivalent
[3] Cat. No. YE-3R, Analytic Lab Accessories, P.O. Box 345, Rockville Centre, NY 11571 or equivalent
[4] Model J6-B, Beckman Instruments, Inc., 2500 Harbor Blvd., Fullerton, CA 92834-3100 or equivalent
[5] Model JS-4.0, Beckman Instruments, Inc. or equivalent
[6] Vortex-2 Genie, Model G-560, Scientific Industries, Inc., 700 Orville Dr., Bohemia, NY 11716 or equivalent

2.2 Reagents/supplies

2.2.1 Round-bottom plate, 96 well[7]

2.2.2 Polystyrene tube, 12 x 75 mm[8]

2.2.3 Conical tube, 50 ml[9]

2.2.4 Pipettes, 2 ml and 25 ml[10]

2.2.5 Reagent reservoir[11]

2.2.6 0.01 M Phosphate buffered saline (PBS)

2.2.6.1 1.33 g sodium phosphate, dibasic, anhydrous (Na_2HPO_4)[12]

2.2.6.2 0.22 g sodium phosphate, monobasic, monohydrate ($NaH_2PO_4 \cdot H_2O$)[13]

2.2.6.3 8.5 g sodium chloride (NaCl)[14]

2.2.6.4 Q.S. to 1000 ml with distilled water (DW).

2.2.6.5 Adjust pH to 7.2-7.6 with 0.1 N sodium hydroxide (NaOH)[15] or 1.0 N hydrochloric acid (HCl).[16]

2.2.6.6 Sterilize by autoclaving at 15 psi, $121^0 \pm 2^0$C for 35 ± 5 min.

2.2.6.7 Store at $4^0 \pm 2^0$C.

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[7] Falcon® 3911, Becton Dickinson Labware, 2 Bridgewater Lane, Lincoln Park, NJ 07035 or equivalent
[8] Falcon® 2058, Becton Dickinson Labware or equivalent
[9] Cat. No. 62.547, Sarstedt, Inc., P.O. Box 468, Newton, NC 28658-0468 or equivalent
[10] Falcon® 7508 and 7585 respectively, Becton Dickinson Labware or equivalent
[11] Costar® 4870, Costar Corp., 1 Alewife Center, Cambridge, MA 02140 or equivalent
[12] Cat. No. S 0876, Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178 or equivalent
[13] Cat. No. S 9638, Sigma Chemical Co. or equivalent
[14] Cat. No. S 9625, Sigma Chemical Co. or equivalent
[15] Cat. No. 925-30, Sigma Chemical Co. or equivalent
[16] Cat. No. 920-1, Sigma Chemical Co. or equivalent

2.2.7 10% Kaolin Suspension

2.2.7.1 10 g kaolin[17] in 100 ml PBS

2.2.7.2 Store at 4⁰ ± 2⁰C.

2.2.8 Alsever's Solution

2.2.8.1 8.0 g sodium citrate ($C_6H_5Na_3O_7 \cdot 2H_2O$)[18]

2.2.8.2 0.55 g citric acid ($C_6H_8O_7 \cdot H_2O$)[19]

2.2.8.3 4.2 g NaCl

2.2.8.4 20.5 g glucose[20]

2.2.8.5 Q.S. to 1000 ml with DW.

2.2.8.6 Sterilize with a 0.22-µm filter.[21]

2.2.8.7 Store at 4⁰ ± 2⁰C.

2.2.9 Chicken red blood cells (RBC) from specific-pathogen-free chickens in an equal volume of Alsever's Solution. Store at 4⁰ ± 2⁰C.

2.2.10 Test Virus. Each manufacturer provides each strain of type A equine influenza virus present in the Test Serial that has been correlated to protection in a host animal immunogenicity trial. Each Test Virus is identified in the Animal and Plant Health Inspection Service (APHIS) filed Outline of Production (OP).

2.2.11 Equine influenza virus reference antiserum (Reference Serum)[22] to serve as a positive control

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[17]Cat. No. K 7375, Sigma Chemical Co. or equivalent
[18]Cat. No. S 4641, Sigma Chemical Co. or equivalent
[19]Cat. No. C 7129, Sigma Chemical Co. or equivalent
[20]Cat. No. G 8270, Sigma Chemical Co. or equivalent
[21]Cat. No. 12122, Gelman Sciences, 600 S. Wagner Rd., Ann Arbor, MI 48106 or equivalent
[22]Reference quantities are available on request from the Center for Veterinary Biologics-Laboratory (CVB-L), P.O. Box 844, Ames, IA 50010

3. Preparation for the assay

3.1 Personnel qualifications/training

Personnel shall have training in hemagglutination (HA) and HI techniques and in standard laboratory procedures.

3.2 Preparation of reagents/control procedures

3.2.1 Upon receipt of the RBC, prepare Washed RBC as follows:

3.2.1.1 Transfer 20 ml of RBC into a 50-ml conical tube.

3.2.1.2 Q.S. to 50 ml with Alsever’s Solution.

3.2.1.3 Mix by inverting several times.

3.2.1.4 Centrifuge for 10 min at 400 X g (1500 rpm in the J6-B centrifuge with a JS-4.0 rotor).

3.2.1.5 Remove supernatant and white blood cell layer by aspirating with a 25-ml pipette.

3.2.1.6 Repeat **Sections 3.2.1.2 through 3.2.1.5** for a total of 3 washes.

3.2.1.7 Store the packed RBC in Alsever’s Solution at $4^0 \pm 2^0$C; use within 1 wk of collection of RBC.

3.2.2 0.5% RBC Suspension for the HA or HI assays. Pipette 500 µl of packed Washed RBC into 100 ml of PBS. Store at $4^0 \pm 2^0$C; use within 1 wk of collection of RBC.

3.2.3 5% RBC Suspension for removal of nonspecific agglutinins from guinea pig sera (GPS). Pipette 100 µl of packed Washed RBC into 1.9 ml of PBS. Store at
$4^0 \pm 2^0$C; use within 1 wk of collection of RBC.

3.2.4 Test Virus Working Dilution. Each Test Virus should contain 8 HA units (HAU) per 50 µl. The Test Virus Working Dilution is determined by an HA assay and verified by back titration for each HI test performed.

3.2.4.1 On the day of test initiation, prepare a series of twofold dilutions of each Test Virus in duplicate from undiluted through 1:2048 in a 96-well, round-bottom plate (**See Template, Section 9.1**).

1. Pipette 50 µl/well of PBS into columns 2-12 with a 12-channel micropipettor.

2. Thaw frozen Test Virus at room temperature (RT) ($23^0 \pm 2^0$C). Add 100 µl of undiluted Test Virus to wells A1 and B1. Additional Test Viruses are tested in duplicate in rows C through F.

3. Transfer 50 µl from column 1 to column 2 with a 12-channel micropipettor; mix (7 ± 2 fills by aspiration and expulsion of the 12-channel micropipettor).

4. Using new pipette tips, continue **Section 3.2.4.1.3** for columns 3-12, transferring 50 µl from the previous well to next well in the column until the dilution sequence is completed. Remove and discard
50 µl from the last column.

5. Add 50 µl of 0.5% RBC to each well containing Test Virus.

6. Place 50 µl of the 0.5% RBC Suspension in each of 3 wells as an RBC Control. Add 50 µl of PBS to each of the 3 RBC Control wells.

7. Mix by tapping the edge of the plate with fingers. Incubate the plate uncovered

for 35 ± 10 min at RT. The optimum time to evaluate results is when distinct buttons have formed in the RBC Control wells.

8. Read the assay as follows: Record "+" for complete agglutination (hazy appearance to the RBC) and "-" for no agglutination (distinct button).

9. One HAU is the highest dilution of virus contained in a 50-µl volume that completely agglutinates the RBC. Calculate the dilution that contains 8 HAU/50 µl by dividing the 1 HAU dilution factor by 8. For example, if the 1:512 dilution contained 1 HAU, then the 1:64 dilution would contain 8 HAU/50 µl or 4 HAU/25 µl. This is the Test Virus Working Dilution.

3.3 Preparation of the sample

3.3.1 Guinea pig sera from test animals may be stored frozen indefinitely at -20^0 ± 2^{0}C. Treated serum may be stored at 4^0 ± 2^{0}C for up to 48 hr, or frozen indefinitely at -20^0 ± 2^{0}C.

3.3.2 Treatment of GPS samples for the removal of nonspecific inhibitors

3.3.2.1 On the day of test initiation, pipette 200 µl of each GPS into individually labeled 12 x 75-mm polystyrene tubes for a total of 12 tubes per Test Serial.

3.3.2.2 Pipette 1.0 ml of 10% Kaolin Suspension (keep kaolin in suspension by shaking) to each tube.

3.3.2.3 Vortex the kaolin/serum mixture tubes on high speed to resuspend the kaolin every 5 min for 20 ± 5 min.

3.3.2.4 Centrifuge at 800 X g (2000 rpm in the J6-B centrifuge with a JS-4.0 rotor) for 20 ± 5 min.

3.3.2.5 Pipette 100 µl 5% RBC to each tube.

3.3.2.6 Pipette 700 µl PBS to each tube.

3.3.2.7 Vortex the tubes on low speed every 5 min for 20 ± 5 min to resuspend the RBC. The kaolin pellet should not be disturbed by this treatment.

3.3.2.8 Centrifuge at 400 X g (1500 rpm in the J6-B centrifuge with a JS-4.0 rotor) for 20 ± 5 min. The RBC pellet will pack down on top of the kaolin pellet.

3.3.2.9 Pour the supernatant from each treated GPS to a clean, labeled 12 x 75-mm polystyrene tube. This is a 1:10 dilution of Treated GPS.

4. Performance of the HI assay

4.1 Pipette 50 µl each of the Treated GPS into the first well of 2 adjacent columns on a 96-well, round-bottom plate for each Test Virus (**See Template, Section 9.2**). This is the Sample Test Plate.

4.2 Pipette 25 µl of PBS into the remaining wells of each column with the 12-channel micropipettor.

4.3 Twofold dilutions are made as follows:

4.3.1 Transfer 25 µl of serum from row 1 to row 2; mix (7 ± 2 fills by aspiration and expulsion of the 12-channel micropipettor).

4.3.2 Using new pipette tips, continue the 25-µl transfer until all dilutions have been made through row 8. Remove and discard 25 µl from the last row.

4.4 Add 25 µl of the Test Virus Working Dilution (4 HAU/25 µl) (**Section 3.2.4**) to each well.

4.5 Virus Back Titration is performed for each Test Virus **(See Template, Section 9.2**).

4.5.1 Add 100 µl of the Test Virus Working Dilution in the first wells of 2 adjacent columns of the Sample Test Plate.

4.5.2 Add 50 µl of PBS in each of the 5 wells in the rows below the first wells of the 2 adjacent columns.

4.5.3 Transfer 50 µl from row 1 to row 2; mix (7 ± 2 fills by aspirating and expulsion of the 12-channel micropipettor).

4.5.4 Using new pipette tips, continue **Section 4.5.3** for rows 3 through 6, transferring 50 µl from the previous wells to the next wells in the column. Remove and discard 50 µl from the last row used.

4.6 Place 25 µl of each GPS in an individual well as an autoagglutination control (GPS Autoagglutination Control). Add 25 µl of PBS to each of these GPS control wells.

4.7 Mix by tapping the edge of the plate with fingers. Incubate the plate uncovered for 30 ± 5 min at RT.

4.8 Place 50 µl of the 0.5% RBC Suspension in each of 3 wells as an RBC Control. Add 50 µl of PBS to each of the
3 RBC Control wells.

4.9 Add 50 µl of 0.5% RBC to each well containing the Treated GPS/Test Virus mixture, the Virus Back Titration, or the GPS Autoagglutination Control.

4.10 Mix by tapping the edge of the plate with fingers. Incubate the plate uncovered for 35 ± 10 min at RT. The optimum time to evaluate results is when distinct buttons have formed in the RBC Control wells.

4.11 Read the agglutination results as follows:

"+" partial to complete agglutination (hazy appearance) indicates absence of antibody in the GPS.

"-" no agglutination (distinct button) indicates presence of antibody in the GPS.

4.12 The HI titer of the GPS is the reciprocal of the highest serum dilution with a distinct button of RBC in at least 1 of the 2 test wells.

4.13 Group the test sera based on the vaccination serial. Determine the geometric mean titer (GMT) for each Test Virus from each group.

5. Interpretation of the HI results

5.1 Validity requirements

5.1.1 The Virus Back Titration must show complete agglutination in the first 3 dilutions, partial to full agglutination in the 4th dilution, and weak partial to no agglutination in the 5th or 6th dilution. The virus endpoint must be 4-8 HAU/50 µl.

5.1.2 For a given fraction, the unvaccinated GPS must remain seronegative at the 1:10 dilution.

5.1.3 None of the GPS Autoagglutination Control wells may exhibit autoagglutination.

5.1.4 The titer of the Reference Serum must fall within ± 1 dilution of its mean titer, as established from a minimum of 10 previously determined titers.

5.1.5 If these validity requirements are not met, the test is **NO TEST** and may be repeated.

5.2 If the GMT of the GPS of a Test Serial for a given fraction in an initial valid test is equal to or greater than the requirements stated in the APHIS filed OP, the Test Serial is **SATISFACTORY**.

5.3 If the GMT of the GPS of a Test Serial in an initial valid test, for a given fraction, is less than the GMT specified in the APHIS filed OP, the Test Serial may be retested in an equivalent number of additional animals as the initial test and the retest GPS tested.

5.3.1 If the GMT of the GPS from all vaccinated GPS of a Test Serial in the initial valid test and the valid retest, for a given fraction, is equal to or greater than the requirements stated in the APHIS filed OP, the Test Serial is **SATISFACTORY**.

5.3.2 If the GMT of the GPS from all vaccinated GPS of a Test Serial in the initial valid test and the valid retest, for a given fraction, is less than the GMT specified in the APHIS filed OP, the Test Serial is **UNSATISFACTORY.**

6. Report of the HI results

Record all test results on the test record.

7. References

7.1 Conrath TB. *Handbook of Microtiter Procedures.* Clinical and Research Applications Laboratory, Cooke Engineering Co., Alexandria, VA, 1972.

7.2 Snedecor GW and Cochran WG. *Statistical Methods*, 6th ed., Iowa State University Press, Ames, IA, Chpt 11, 1967.

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8. Summary of revisions

This document was rewritten to meet the current NVSL/CVB QA requirements, to clarify practices currently in use in the CVB-L, and to provide additional detail. The following is a list of the significant changes made from the previous protocol:

8.1 Meet the requirements stated in 9 CFR, Part 113.217

8.2 Added a Reference Serum Positive Control

8.3 Removed the requirement for male chickens as the source of chicken RBC

8.4 Added the ability to store GPS frozen until tested

8.5 Added GPS Autoagglutination Control

9. Appendices

9.1 Appendix I

Standardization of Test Viruses Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	A 1											
B	A 1											
C	A 2											
D	A 2											
E	A 3											
F	A 3											
G	UN	1:2	1:4	1:8	1:16	1:32	1:64	etc.	etc.	etc.	etc.	etc.
H	RBC	RBC	RBC									

A 1= Type A Influenza Isolate No. 1, etc. RBC= RBC Control

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9.2 Appendix II

Sample Test Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A 1:10	GPS 1	GPS 1	GPS 2	GPS 2	GPS 3	GPS 3	GPS 4	GPS 4	GPS 5	GPS 5	BT	BT
B 1:20											1:2	1:2
C 1:40											1:4	1:4
D 1:80											1:8	1:8
E 1:160											1:16	1:16
F 1:320											1:32	1:32
G 1:640											RBC	RBC
H 1:1280											RBC	RBC

GPS= Treated Guinea Pig Serum
BT= Back Titration of the Test Virus Working Dilution
RBC= RBC Control